Synthetic seed technology for encapsulation and regrowth of in vitro-derived shoot-tips and somatic embryos of *Asparagus officinalis* L.

Tecnología de semillas sintéticas para la encapsulación y el recrecimiento de puntas de brotes y embriones somáticos de *Espárrago officinalis L.*

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ABSTRACT

Apical buds obtained from Asparagus plant vitro culture and somatic embryos obtained from stem cultivation, explants in MS medium supplemented with mg^{1L} 1, 2, 4-D and mg^{1L} 1 and Kinetin have been used in this research to produce artificial seeds. We encapsulated apical buds and somatic embryo using 2% sodium alginate and calcium chloride to prepare the artificial seeds. We placed artificial seeds at room temperature (about 25 ° C), in the cold, the temperature of 4 ° C and -18 ° C for different times (15,30,60,90 days) and evaluated the growing power of these seeds in MS and ½MS mediums for further investigations about the viability of seeds. The highest conversion percentage of seedlings in encapsulated embryos (70.01) was related to seed harvested from embryos treated with BA and the highest conversion percentage of seedlings in apical buds (96.54) was obtained from cultivated untreated seeds in MS medium. Encapsulated arteries and buds maintained germination energy and viability with increasing storage time after 90 days of storage at 4 and 25 ° C despite viability reduction while un-capsulated embryos and buds completely lost viability after 60 days of storage at 4 and 25 ° C and seeds stored at -18 ° C completely lost viability after 15 days of storage. In general, the percentage of seed germination and conversion to seedling is higher in seeds cultivated in MS medium compared to seeds cultivated in ½MS medium.

Keywords: Alginate matrix; Cold storage; Encapsulation; shoot tips; somatic embryos; Synthetic seed

RESUMEN

En esta investigación se han utilizado yemas apicales obtenidas de cultivo vitro vegetal de espárragos y embriones somáticos obtenidos del cultivo de tallos, explantes en medio MS suplementado con mg^{1L} 1, 2, 4-D y mg^{1L} 1 y Kinetin para producir semillas artificiales. Encapsulamos yemas apicales y embriones somáticos utilizando alginato de sodio al 2% y cloruro de calcio para preparar las semillas artificiales.

Colocamos semillas artificiales a temperatura ambiente (alrededor de 25 ° C), en el frío, la temperatura de 4 ° C y -18 ° C para diferentes tiempos (15,30,60,90 días) y evaluamos el poder de crecimiento de estas semillas. en medios MS y ½MS para futuras investigaciones sobre la viabilidad de las semillas. El mayor porcentaje de conversión de plántulas en embriones encapsulados (70.01) se relacionó con la semilla recolectada de embriones tratados con BA y el mayor porcentaje de conversión de plántulas en yemas apicales (96.54) se obtuvo de semillas cultivadas sin tratar en medio MS. Las arterias y las yemas encapsuladas mantuvieron la energía de germinación y la viabilidad con un mayor tiempo de almacenamiento después de 90 días de almacenamiento a 4 y 25 ° C a pesar de la reducción de la viabilidad, mientras que los embriones y las yemas no encapsulados perdieron completamente la viabilidad después de 60 días de almacenamiento a 4 y 25 ° C y las semillas almacenado a -18 ° C perdió completamente la viabilidad después de 15 días de almacenamiento. En general, el porcentaje de germinación de semillas y conversión a plántula es mayor en semillas cultivadas en medio MS en comparación con semillas cultivadas en medio ½MS.

Palabras llave: Matriz de alginato; Almacenamiento en frio; Encapsulamiento; consejos de lanzamiento; embriones somáticos; Semilla sintética

1. INTRODUCCIÓN

Asparagus officinalis L. is commercially the most important specie of Asparagus and is an expensive and very valuable plant. The growth rate of *Asparagus officinalis* L. is low through traditional methods and the proliferation of *Asparagus officinalis* L. using seed is not efficient due to low germination rate of this plant and its time consuming reproducing process by dividing the crown of the plant (Ørnstrup, 1997). So the micro propagation is the perfect solution for this problem. somatic embryogenesis has been considered has the most effective method for micro propagation of plants (Raemakers, Jacobsen, & Visser, 1995). Asparagus is the first specie of monocots regenerated through somatic embryogenesis and somatic embryos is suitable for asexual reproduction of Asparagus (Christou, 1988). Somatic embryos in Asparagus has been done for the first time by wilmer and Hellendoorn in 1968 (Wilmar & Hellendoorn, 1968). Embryo genic callus has been reported many *A.officinalis* L. cultivars but all genotypes do not have the ability to form embryogenic callus (Delbreil & Jullien, 1994).

People who have acted to produce embryos somatic cells for the first time have probably paid attention to artificial seed production, however the concept of artificial seed was not clear until the late 1970s and the first idea to create artificial seed was expressed in 1977 by Murashige, he pointed out that artificial encapsulated somatic embryo can be used as a natural seed (Reddy, Murthy, & Pullaiah, 2012).

Quality and survival of Somatic embryo are among limiting factors in the development of artificial seeds. Production of high quality and uniform embryos which is extremely important in artificial seed production is only limited to a small number of products such as carrots and hay. That is why using monopole structures such as apical buds and lateral buds is also taken into consideration in the past few years. Kamada stated in 1985 that any type of plant explant with germination can be used to produce synthetic seeds (Katouzi, Majd, Fallahian, & Bernard, 2011). After that several scholars used reproducible masses in vitro culture conditions such as lateral buds of such as treetops, sprouts, onions or any meristematic tissue to produce synthetic seeds. Therefore, artificial seeds include lateral buds of stem, apical shoots and mass of an embryo or any part of the plant meristematic tissue in addition to encapsulated somatic embryo which can be converted into a complete plant in natural or vitro conditions (Bapat & Mhatre, 2005). Artificial seed technology is an outstanding technique which is used to reproduce and maintain the plan and has been a lot of plants . Artificial seed technology is currently a very valuable method for clonal micropropagation of different plant species in large scale.

In 1994, Ghosh and Sen produced artificial seed from somatic embryo of *Asparagus cooperi* plant and Mamiya and Sakamoto invented a new method for producing encapsulated units (artificial seeds) in *Asparagus officinalis* L., these units were produced from somatic embryo and had strong germination and growth ability in non-sterile soils. According to our information, there are no reports of artificial seed production of apical buds of Asparagus. We have evaluated the effect of different storage periods at different temperatures on the survival and germination and complete seedling production from artificial seed produced from apical buds and Somatic embryos.

2. MATERIALS AND METHODS

2.1. Plant material and preparation of explants

We prepare Asparagus plant seeds from the Pakan Bazr Institute in Isfahan. These seed were among Mary Washington species. We well-washed the prepared seeds with water. We placed the seeds in 70 degrees' ethanol under laminar hood for one minute after outward decontamination and washed three times by sterile distilled water and then placed them in 2% sodium hypochlorite solution for fifteen minutes and washed them three times (5 minutes each) to completely remove Sodium hypochlorite from the seeds. We then cultured sterile seeds in MS basal medium (Murashige & Skoog, 1962) and placed them in light conditions of 16 hours of light and 8 hours of darkness at 25 ± 1 °C. The seeds germinated after day 9-11 and sterile seedlings were formed after 20 days of culture which were used for the preparation of sterile explants which are used for the production of somatic embryo. We placed 5-7 mm pieces of stem in MS solid medium supplemented with 1 mg^{1L} 2, 4-D + 1 mg^{1L} Kinetin and stored them in the germinator in darkness and at a temperature of 25 ± 1 (Figure 1) and explant was performed every three weeks. We moved produced embryo callus to hormone-free MS base medium after three explants and placed embryogenic callus which have formed embryo suspension in MS medium containing 250 mg plus casein and 30 g of sucrose in the dark and on a shaker for 20 days after three weeks and we used embryos which were in the final stages of embryonic for artificial seed after 20 days after examining under the stereomicroscope. After about 45 days from planting sterile seeds in MS medium putting them in proper light conditions inside the germinator, the plants resulting from seeds had suitable germinates for artificial seed preparation. Apical buds were removed to the length of 2-3 mm and were used in the preparation of artificial seed. We treated some of the somatic embryos with BA and treated some apical buds with NAA in order to evaluate their effect on conversion of seeds to seedling. We adjusted the PH of the medium to 5.6 to 5.8 before adding agar (8 g per liter) and used autoclave under pressure (1.1 kg per cubic centimeter) and a temperature of 120 °C for 20-15 minutes for sterilizing medium.
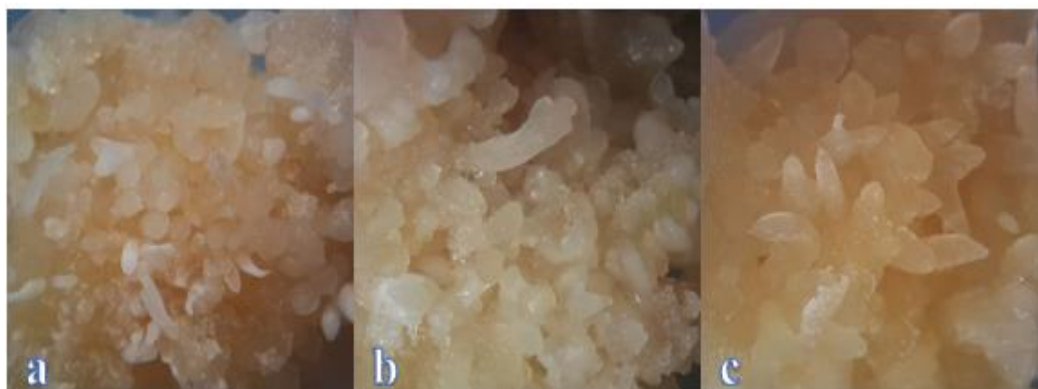
Figure 1: a, b, c: somatic embryos produced in solid medium MS supplemented with 1 mg^{1L} 2, 4-D + 1 mg^{1L} Kinetin and sub cultured in basic MS hormone-free medium.

2.2. Encapsulation of somatic embryos and apical buds

We separately mixed single somatic embryos and apical buds in a solution of 2% sodium alginate in MS medium and then added embryos with sodium alginate to the mM100, $CaCl_2.2H_2O$ solution which had previously been autoclaved using sampler and placed it in the same solution on the shaker for thirty minute and then washed it for 20 minutes using distilled water (Figure 2 a, f). The used solution was separately autoclaved at 120 ˚C for 15-20 minutes at a pressure (1.1 kg per cubic centimeter) after adjustment of the pH to 5.6 ± 0.2.

2.3. Conversion of somatic embryos obtained from artificial seed and Apical buds to seedling

We cultured un-capsulated Arteries and apical buds and encapsulated Arteries and apical buds and Embryos treated with BA and encapsulated apical buds treated with NAA in MS and ½ MS mediums and evaluated the percentage of germination and conversion to seedling. In the seeds obtained from encapsulated apical buds, initially stems were removed from the alginate capsules and Roots started growing after stem growth and about 15-20 days after planting (Figure 2 b, c, d, and e). In seeds obtained from embryos, seeds germinated after 7-10 days after sowing (Figure 2 g, h, i) we placed the prepared seeds at different temperatures including -18, 4 and 25°C in another experiment and evaluated their germination and growth after 15, 30, 60 and 90 days and took them out of sterile conditions after formation of artificial seedlings and slowly transferred them to pots containing peat and sand (ratio 1: 1) which had already been sterile and covered its surroundings with covers and watered with MS medium until the first week and gradually created holes in the cover so that so that the plant can start the stage of compromise with the external medium and be compatible with drought and water irrigation mediums, we ultimately transferred sample to the greenhouse after a month.

Figure 2. Artificial seed produced from somatic arteries and apical buds of *Asparagus officinalis* L. and germination and growth in the MS medium a) encapsulated Apical buds in alginate beads b, d) new stems coming out of artificial seed produced from apical buds c, e) Growth and of roots of artificial seed produced coming out of apical buds f) encapsulated embryos in alginate beads g, h and i) Germination of seeds and Growth and roots and stems coming out of artificial seed produced from somatic embryos.

2.4. Statistical analysis

We measured the results of conversion of un- capsulated arteries and apical buds and encapsulated arteries and apical buds without treatment and embryos treated with BA and encapsulated apical buds treated with NAA (Stored and un-stored) after 4 weeks of culture in the medium of MS and ½MS. 40 artificial seeds were placed in 4 culture dishes in each experiment and each experiment was performed three times. The results of each test and repetitiveness of it was done after calculations in SPSS (Ver.22) software and ANOVA test with the probability level of $P \leq 0.05$, comparisons and graphs were plotted using Excel 2013.

3. RESULTS AND DISCUSSION

As shown in Table 1, the conversion percentage of apical buds without cover, covered apical buds with no treatment and covered apical buds treated with NAA which have been cultured immediately and without storage in MS and ½MS mediums was respectively 55, 53.02 and 85.05 percent and their conversion percentage reduced gradually by increasing storage period at 4 ° C in a way that after 30 days of storage at 4 °C, the conversion percentage of apical buds without cover, covered apical buds with no treatment and covered apical buds treated with NAA cultured in MS medium was respectively 21.72 and 50.01 and 78.51 percent and was 19.96 and 46.13 and 75.18 percent in ½MS medium. Bapat et al. (1987) reported in this regard that encapsulated lateral buds of mulberry have fully maintained their viability after 45 days of storage at 4 ° C and will convert into a complete plant (Bapat, Mhatre, & Rao, 1987). Also, according to

the results of research of Rady and Hanafi (2004), conversion percentage of seeds of apical buds of Gypsophila *paniculata* L will reduce by increasing storage time (Rady & Hanafy, 2004). Covered apical buds showed higher resistance compared to uncoated types in this research. These differences have been attributed to the role of the protection afforded by the alginate capsules. The germination percentage of uncovered apical buds reached to zero after 60 days and these seeds lost their viability. However, covered apical buds without treatment and covered apical buds treated with NAA cultivated in MS and ½MS mediums had maintained their ability but the percentage of conversion to seedling also decreased in them by increasing storage period. The obtained results are in line with the results of the studies of Results Singh (2009) who worked on Spilanthes acmella plant (Singh et al., 2009) and Salehi Katouzi et al. (2011) who worked on *Helianthus annuus* L. plant (Katouzi et al., 2011). The germination percentage of seeds obtained from buds without treatment and buds without cover in seeds obtained from buds was about the same percentage of germination of seed obtained from bud treated with NAA and a higher percentage of buds treated with NAA have formed roots during growth and conversion to seedling and have produced a complete plant while a lower percentage of untreated buds produced roots

Table 1. Effects of different storage periods at the temperature of 4 °C on conversion percentage to complete plant in apical buds without cover, covered apical buds without treatment and covered apical buds without treated with NNA cultured in MS and ½MS (Results have been shown in form of $\overline{X} \pm EXE$ in three repeats, different letters show the significance and similar letters show the insignificance of differences)

Seed obtained from buds treated with NAA	seeds obtained from bud without treatment	Uncovered buds	Type of medium in which seeds have been cultivated	Storage period of seeds
85.05 ± 0.11 a	53.02 ± 0.11 b	55.00 ± 0.06 b	½MS	0
90.40 ± 0.05 a	57.05 ± 0.05 b	59.62 ± 0.07 b	MS	0
80.07 ± 0.07 a	50.04 ± 0.04 b	41.05 ± 0.09 c	½MS	15
84.13 ± 0.07 a	54.14 ± 0.07 b	44.02 ± 0.08 c	MS	15
75.18 ± 0.44 a	46.13 ± 0.07 b	19.96 ± 0.08 c	½MS	30
78.51 ± 0.07 a	50.01 ± 0.09 b	21.72 ± 0.08 c	MS	30
64.01 ± 0.08a	39.94 ± 0.04 b	0.00 ± 0.00 c	½MS	60
67.03 ± 0.03 a	42.93 ± 0.03 b	0.00 ± 0.00 c	MS	60
51.54 ± 0.10 a	32.28 ± 0.06 b	0.00 ± 0.00 c	½MS	90
54.24 ± 0.04 a	35.04 ± 0.06 b	0.00 ± 0.00 c	MS	90

According to Table 2, conversion percentage of apical buds without cover, covered apical buds with no treatment and covered apical buds treated with NAA gradually reduced by increasing storage period at 25 °C similar to seeds stored at 4 °C. coated Apical buds showed a higher resistance compared to different uncovered types during storage period. The germination percentage of uncovered apical buds reached to zero after 60 days and these seeds lost their viability. Based on the results of the study of Islam and Bari (2012) on Mentha arvensis plant, encapsulated apical buds will lose their viability after 30 days of storage and lose their viability after 45 days of encapsulated nodal explants storage at the temperature of 20 ± 2 °C (Islam & Bari, 2012). On this basis, the viability of seeds obtained from Asparagus is higher than Mentha arvensis plant in the long-term storage at 25°C.

Table 2: Effects of different storage periods at the temperature of 25 °C on conversion percentage to complete plant in apical buds without cover, covered apical buds without treatment and covered apical buds without treated with NNA cultured in MS and ½MS (Results have been shown in form of $\overline{X} \pm EXE$ in three repeats, different letters show the significance and similar letters show the insignificance of differences)

Seed obtained from buds treated with NAA	seeds obtained from bud without treatment	Uncovered buds	Type of medium in which seeds have been cultivated	Storage period of seeds
85.05 ± 0.11 a	53.02 ± 0.11 b	55.00 ± 0.06 b	½MS	0
90.40 ± 0.05 a	57.05 ± 0.05 b	59.62 ± 0.07 b	MS	0
43.48 ± 0.10 a	27.06 ± 0.06 b	20.74 ± 0.05 b	½MS	15
45.05 ± 0.10 a	29.00 ± 0.11 b	22.50 ± 0.08 b	MS	15

33.40 ± 0.05 a	21.06 ± 0.09 b	10.04 ± 0.06 c	½MS	30
34.45 ± 0.10 a	22.42 ± 0.04 b	11.07 ± 0.09 c	MS	30
21.04 ± 0.04 a	13.50 ± 0.05 b	0.00 ± 0.00 c	½MS	60
22.04 ± 0.04 a	14.01 ± 0.07 b	0.00 ± 0.00 c	MS	60
8.16 ± 0.10 a	5.61 ± 0.07 b	0.00 ± 0.00 c	½MS	90
8.51 ± 0.04 a	5.99 ± 0.06 b	0.00 ± 0.00 c	MS	90

Seeds obtained from apical buds stored at 4 °C had higher viability and conversion percentage than seeds stored at 25 °C and the difference in the conversion ability was significant. The conversion ability of seed storage at 25 °C will have greater reduction compared to 4 °C (Figure 1). These results are in line with reports of Islam and Bari (2012) about the Mentha arvensis plant.
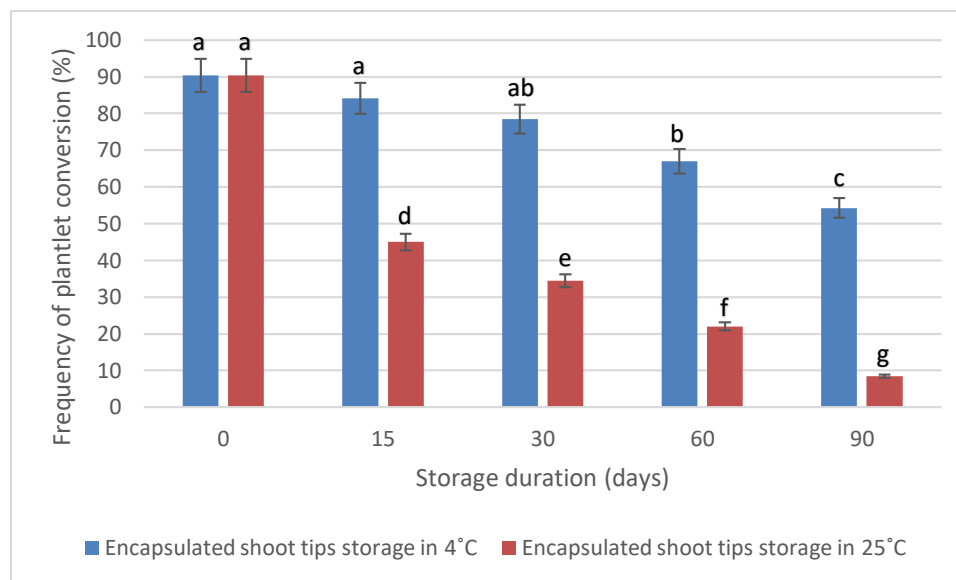

Figure 1. The effect of different storage temperatures on the ability to convert to seedlings in seeds obtained from apical buds cultured in MS.

According to table 3, conversion percentage of Embryo without cover, seeds obtained from embryo without treatment and seeds obtained from embryo treated with BA which have been cultured immediately and without storage in MS and ½MS mediums was same and there was no significant difference between them. This matter shows that the material used to encapsulate the embryos does not have inhibitor effect on germination and growth of seeds. The conversion percentage of seeds dropped gradually by increasing storage period in a way that it reduced from 54.07 in the control sample to 24.05 in samples stored for 90 days in seeds obtained from embryo treated in ½MS medium and reached to 29.96 from 68.01 in seeds obtained from embryo without treatment cultured in MS medium. Conversion power reached to 31.27 from 70.01 in seeds cultivated in MS medium in seeds obtained from embryo treated with Ba and reduced to 25.10 from 55.96 in the ½MS medium. According to the study of Singh and Chand (2010) on Dalbergia sissoo plant (Singh, Rai, Asthana, & Sahoo, 2010), the germination and seedling conversion of encapsulated somatic embryos stored at 4 °C will reduced and based on the report of Bazinet et al (1992) the regeneration rate of Daucus carota the plant after storage in cold will reduced due to loss of viability which are all in line with the results of our study (Bazinet et al., 1992). Storage of somatic embryos covered by Sodium alginate has only been successfully completed in some species. According to Gosh and Sung (1994), only 8.33% of encapsulated embryos have germinated after 90 days of storage at a temperature of 2 °C (Ghosh & Sen, 1994). Ipekci and Gozukirmizi (2003) observed in a study on plants of Paulownia elongate that the germination percentage of seeds obtained from embryo was 32.40 percent after 60 days of storage at 4 °C (Ipekci & Gozukirmizi, 2003). Similar results have been observed in the researches of Redenbaugh et al. (1987) Alfalfa on artificial seed of Alfalfa plant

(Redenbaugh, Slade, Viss, & Fujii, 1987) and Muralidharan and Mascarenhas (1995) on Eucalyptus citrisdora plant (Muralidharan & Mascarenhas, 1995). The covered embryos have shown higher viability than uncovered types during the storage period in this research. The germination percentage of uncovered embryos reached to zero after 60 days and these seeds lost their viability while, the covered embryos have maintained their viability despite the reduced ability to convert to the seedling. In general, the conversion percentage to seedling is higher in seed produced in MS medium than ½MS medium. According to the results of Singh and Chang (2005), regenerate is not done in uncovered Somatic embryos stored at 4 °C (Provenzano et al., 2005).

Table 3: Effects of different storage periods at the temperature of 4 ° C on conversion percentage to complete plant in embryo without cover, seeds obtained from embryo without treatment and seeds obtained from embryos treated with BA cultured in MS and ½MS (Results have been shown in form of $\bar{X} \pm EXE$ in three repeats, different letters show the significance and similar letters show the insignificance of differences)

Covered and treated embryo	Covered embryo without treatment	embryo without cover	Type of medium in which seeds have been cultivated	embryo treated with BA
55.96 ± 0.09 a	54.07 ± 0.10a	54.40 ± 0.05 a	½MS	0
70.01 ± 0.07 a	68.01 ± 0.07 a	68.26 ± 0.09 a	MS	0
54.44 ± 0.10a	52.58 ± 0.22 a	38.46 ± 0.2 b	½MS	15
68.54 ± 0.06 a	66.35 ± 0.07 a	48.56 ± 0.09 b	MS	15
49.29 ± 0.04 a	47.55 ± 0.10a	19.54 ± 0.03 b	½MS	30
62.03 ± 0.04 a	60.07 ± 0.07 a	24.58 ± 0.04 b	MS	30
39.98 ± 0.08 a	38.28 ± 0.10 a	0.00 ± 0.00 b	½MS	60
49.58 ± 0.08 a	48.06 ± 0.08 a	0.00 ± 0.00 b	MS	60
25.10 ± 0.06 a	24.05 ± 0.05 a	0.00 ± 0.00 b	½MS	90
31.27 ± 0.11 a	29.96 ± 0.09 a	0.00 ± 0.00 b	MS	90

As shown in Table 4, conversion percentage of uncovered embryo, seeds obtained from embryo without treatment and seeds obtained from embryo treated with BA which have been cultured immediately and without storage in MS and ½MS mediums was same and there was no significant difference between them. Conversion percentage of uncovered embryo, seeds obtained from embryo without treatment and seeds obtained from embryo treated with BA dropped gradually and with increasing storage period at 25 °C similar to seeds stored at 4 °C. covered embryos showed higher resistance during the storage period compared to uncovered types. The germination percentage of uncovered embryos reached to zero after 60 days of storage at 25 °C and these seeds lost their viability.

Table 4: Effects of different storage periods at the temperature of 25 °C on conversion percentage to complete plant in embryo without cover, seeds obtained from embryo without treatment and seeds obtained from embryos treated with BA cultured in MS and ½MS (Results have been shown in form of $\bar{X} \pm EXE$ in three repeats, different letters show the significance and similar letters show the insignificance of differences)

Covered and treated embryo	Covered embryo without treatment	embryo without cover	Type of medium in which seeds have been cultivated	embryo treated with BA
55.96 ± 0.09 a	54.07 ± 0.10 a	54.40 ± 0.05 a	½MS	0
70.01 ± 0.07 a	68.01 ± 0.07 a	68.26 ± 0.09 a	MS	0
36.56 ± 0.09 a	34.91 ± 0.08 a	26.36 ± 0.06 b	½MS	15
45.52 ± 0.06 a	44.10 ± 0.10 a	32.39 ± 0.05 b	MS	15
28.09 ± 0.05 a	26.71 ± 0.11 a	12.89 ± 0.06 b	½MS	30
36.13 ± 0.07 a	35.02 ± 0.08 a	16.44 ± 0.03 b	MS	30
17.94 ± 0.03 a	16.53 ± 0.06 a	0.00 ± 0.00 b	½MS	60
24.17 ± 0.04 a	23.18 ± 0.09 a	0.00 ± 0.00 b	MS	60
7.30 ± 0.02a	6.69 ± 0.11 a	0.00 ± 0.00 b	½MS	90
10.00 ± 0.05 a	9.22 ± 0.05 a	0.00 ± 0.00 b	MS	90

Seeds obtained from embryo stored at 4 °C had higher viability and conversion percentage compared to seeds stored at 25 °C and the conversion ability of seed storage at 25 °C will have greater reduction

compared to 4 °C by increasing storage period (Figure 2). These results are in line with reports of Islam and Bari (2012) on Mentha arvensis plant.
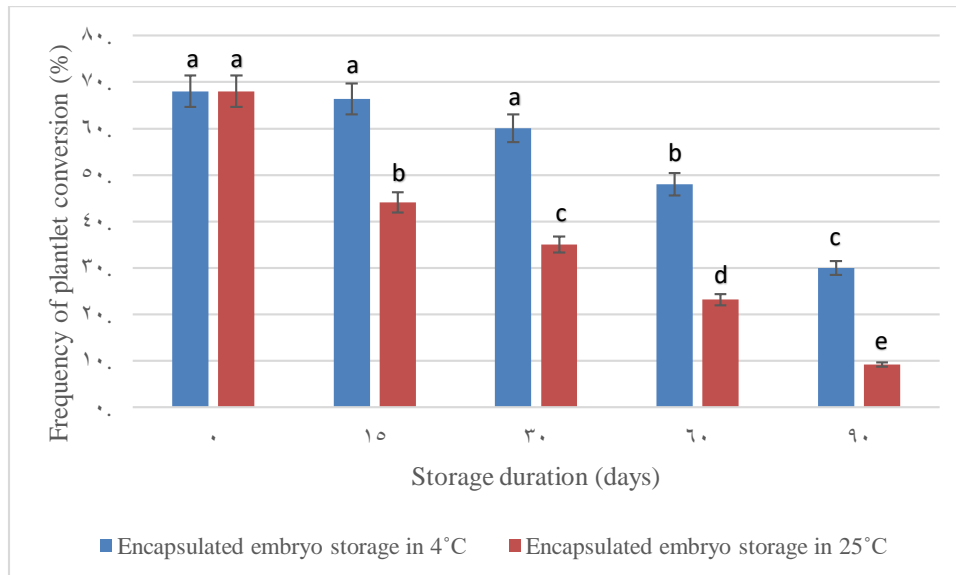

Figure 2. the effect of different storage temperatures on the ability to convert to seedlings in seeds obtained from embryo cultured in MS.

4. CONCLUSIONES

Based on the results of this experiment, conversion percentage of all types of synthetic seeds obtained from buds, reduced, by increasing the storage temperature of seeds from 4 °C to 25 °C. Also, by increasing the storage time of seeds at different temperatures, the conversion rate decreases. In the synthetic seeds obtained from embryo, like the synthetic seeds obtained from buds, by increasing the storage temperature and storage period, the conversion ability decreases. And in both cases, the seeds stored at low temperatures were more durable and had a higher conversion rate.

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